Induced hyperketonemia affects the mammary immune response during lipopolysaccharide challenge in dairy cows

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DOI: https://doi.org/10.3168/jds.2013-7222

Interpretive Summary:

Induced hyperketonemia affects the mammary immune response during lipopolysaccharide challenge in dairy cows. *By Zarrin et al*. Effects of an induced hyperketonemia through beta-hydroxybutyrate (BHBA) infusion and an additional intramammary lipopolysaccharide (LPS) challenge on immune response in liver and mammary gland were studied in mid-lactating dairy cows. BHBA infusion caused increased acute phase protein mRNA abundance in the mammary gland but not in the liver. The LPS-related increase of somatic cell counts (SCC) was less pronounced whereas mRNA abundance of IL-8, IL-10 increased more in the response to LPS in the group receiving BHBA infusion than the control animals. The results demonstrate that elevated BHBA has most likely a direct effect on the susceptibility of mastitis.

HYPERKETONEMIA AFFECTS IMMUNE RESPONSE IN DAIRY COWS

Induced hyperketonemia affects the mammary immune response during lipopolysaccharide challenge in dairy cows

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ABSTRACT

Metabolic adaptations during negative energy and nutrient balance in dairy cows are supposed to cause impaired immune function and hence increased risk of infectious diseases including mastitis. Characteristic adaptations mostly occurring in early lactation are an elevation of plasma ketone bodies and free fatty acids (NEFA) and diminished glucose concentration. The aim of this study was to investigate effects of elevated plasma beta-hydroxybutyrate (BHBA) at simultaneously even or positive energy balance and thus normal plasma NEFA and glucose on factors related to the immune system in liver and mammary gland of dairy cows. In addition, the effect of elevated plasma BHBA and intramammary lipopolysaccharide (LPS) challenge on the mammary immune response was investigated. Thirteen dairy cows were either infused with BHBA (HyperB, n=5) to induce hyperketonemia (1.7 mmol/L), or with a 0.9 % saline solution (NaCl, n=8) for 56 h. Two udder quarters were injected with 200 µg LPS after 48 h of infusion. Rectal temperature (RT) and somatic cell counts (SCC) were measured before, at 48 h after the start of infusions, and hourly during LPS challenge. The mRNA abundance of factors related to the immune system was measured in hepatic and mammary tissue biopsies one week before, and 48 h after the start of the infusion, and in mammary tissue additionally at 56 h of infusion (8 h after LPS administration). At 48 h of infusion in HyperB, the mRNA abundance of serum amyloid A (SAA) in the mammary gland was increased ($P<0.01$), and that of haptoglobin (Hp) tended to be increased ($P= 0.07$). Rectal temperature, SCC, and mRNA abundance of candidate genes in the liver were not affected by the BHBA infusion until 48 h. During the following LPS challenge RT and SCC increased in both groups ($P<0.01$). However, SCC increased less in HyperB than in NaCl ($P<0.01$). Quarters infused with LPS showed a more pronounced increase of mRNA abundance of IL-8, IL-10 in HyperB than in NaCl ($P<0.05$). The results demonstrate that an increase of plasma BHBA up-regulates acute phase proteins in the mammary gland. In

response to intramammary LPS challenge elevated BHBA diminishes the influx of leukocytes from blood into milk possibly via modified cytokine synthesis. Results indicate that increased ketone body plasma concentrations may play a crucial role in the higher mastitis susceptibility in early lactation.

INTRODUCTION

Since decades dairy cows have been selected for high milk production. Concurrently with the increase in milk production, the incidence of infectious diseases increased (Simianer et al., 1991; Syvajarvi at al., 1986; Uribe at al., 1995). Mainly during the first weeks of lactation cows experience an immunosuppression and high susceptibility of infectious diseases (Smith et al., 1985; Hogan et al., 1989; Goff, 2006) which is supposed to be due to the metabolic adaptations to negative energy balance (NEB) (Suriyasathaporn et al., 2000; van Dorland., 2009). Low plasma glucose concentrations, and elevated plasma NEFA and ketone body concentrations, specifically of BHBA, are the characteristic changes during NEB (Bobe et al., 2004; van Dorland et al., 2009; Gross et al., 2011). Increased plasma BHBA concentrations above 1200 µmol/L are considered as a metabolic disorder (Ospina et al., 2010) defined as subclinical ketosis (Duffield et al., 2009).

The occurrence of mastitis is determined by a number of pathogen, animal, and environmental factors (Burvenich et al., 2007). Among the animal factors high plasma BHBA concentration has negative influence on the susceptibility of mastitis and course of disease (Heyneman et al., 1990; Van Werven et al., 1997; Oltenacu and Ekesbo 1994). In vitro, growth of bacteria obtained from LPS-challenged quarters in hyperketotic and normal dairy cows showed that high plasma BHBA concentrations had a high positive correlation with the severity of mastitis induced by alive *E. coli* (Kremer et al., 1993). In addition, elevation of plasma BHBA concentration decreased in vitro chemotaxis and microbial killing in human (McMurray et al.,

1990) as well as respiratory burst activity of bovine neutrophils (Hoeben et al., 1997). Cytokine production was reduced after bacterial infection in ketotic dairy cows (Kandefer-Szerszen et al., 1992; Filar et al., 1992). However, elevated BHBA occurs mostly concomitantly with other metabolic and endocrine changes (Kessel et al., 2008; Gross et al., 2011), and the immunosuppressive effect cannot be exclusively ascribed to the ketone bodies. Results of exclusive BHBA effects on the immune system are available from in vitro studies. Thus, in the presence of BHBA a decreased phagocytotic activity of milk polymorphonuclear leukocytes was demonstrated (Klucinski et al., 1988), as well as a reduced chemotactic capacity of bovine blood leukocytes (Suriyasathaporn et al., 1999). The role of BHBA in the immunosuppression in vivo is not fully understood (Burton et al., 2005). Information about the effects of hyperketonemia isolated from other metabolic changes on the immune response and SCC in hyperketotic cows is rare, and the effects of long term hyperketonemia have not been investigated in dairy cows. The objective of this work was to investigate effects of a 48 h elevation of plasma BHBA through BHBA infusion on parameters related to the immune system in liver and mammary gland, and to study the mammary immune response to LPS challenge during elevated BHBA for additional 8 h.

MATERIAL AND METHODS

Animals and Management

All procedures involving animals followed the Swiss Animal Protection Law and were approved by the Federal Veterinary Office in Switzerland. Thirteen non pregnant Holstein dairy cows in diestrus with a parity of 3.5 ± 0.10, at 28 ± 0.3 (MEAN ± SD) weeks in milk were selected. Cows in mid lactation were chosen as the best possible model for investigation of the specific effect of BHBA infusion without the comprehensive endocrine and metabolic changes that occur during the period of highest milk production in early lactation. The cows were free of mastitis and other infectious diseases which was confirmed by a routine blood

109 glutaraldehyde coagulation test (Sandholm, 1976), and by measuring milk SCC (DeLaval cell
110 counter DCC, DeLaval International AB, Tumba, Sweden), which had to be less than 150 x
111 10^3 cells/mL in all four quarters at the start of the experiment. The validity and repeatability
112 of Delaval cell counter was previously shown (Sarikaya and Bruckmaier, 2006).
113 Animals were moved to the experimental tie stall for adaptation to housing and feeding
114 conditions two weeks before the start of the experiment. Both groups were fed ad libitum with
115 hay (dry matter [DM] content, 890g/kg of fresh matter [FM]; on DM basis, consisting of 153
116 g of crude protein [CP]/kg, 235.0 g of crude fiber [CF]/kg, and 5.7 MJ of NEL/kg). In
117 addition they received a protein- and energy-rich concentrate (23.5% barley, 14.0% oats,
118 20.0% wheat bran, 17.0% soybean expeller, 15.0% linseed meal, 0.6% salt, 2.2% carbonate of
119 lime, 0.4% calf rearing feed premix , 4.0% molasses and 3.0% by pass fat, DM content, 881
120 g/kg of FM; on DM basis, consisting of 217 g of CP/kg, 73.9 g of CF/kg, and 7.6 MJ of
121 NEL/kg) twice daily according to individual milk production. In addition, minerals (50 g/
122 cow) were supplied once a day. Animals had access to fresh water ad libitum. Milking was
123 performed twice daily at 0530 h and 1600 h.
124
125 ***Experimental design and treatments***
126 Cows were randomly allocated to one of two infusion treatment groups as described earlier
127 (Zarrin et al., 2013). In brief the treatments involved an intravenous Na-DL-β-OH-butyrate
128 infusion to obtain plasma BHBA concentration between 1.5 to 2.0 mmol/L (HyperB, n=5)
129 comparable to those in spontaneous hyperketonemia, and a saline (0.9 %) infusion (NaCl, 20
130 mL/h, n=8) as control group. One day before the infusions started, cows were fitted with
131 indwelling intravenous catheters (Cavafix® Certo® Splittocan®, B. Braun Melsung AG,
132 Germany) with a length of 32 cm and a diameter of 16 G in both jugular veins. The infusions
133 started at 0900 on day 1 and continued to 1700 two days later. At 48 h of infusions, each of
134 two udder quarters was injected with 200 µg of LPS *Escherichia coli* serotype 026:B6 (#

L8274; Sigma-Aldrich) as LPS quarter, and 0.9 % NaCl solution were injected in two udder quarters as control. Details were described by Vernay et al. (2012).

Data collection and sampling

Blood samples. Baseline blood samples at 1 wk and at 2 h before the start of the infusion were taken between milking and feeding at 0730 h. Sampling was continued at least hourly during the 56 h infusion period. During the experimental infusions blood samples were obtained from the contralateral jugular catheter, which was not used for the infusion, by using tubes containing tri-potassium-EDTA. Plasma was separated by centrifugation at 3000 x g for 20 minutes, and stored at -20 °C until analyses. Every 15 min during the first 2 h and every hour during the entire infusion period additional small blood samples (1mL) were analyzed immediately for adjustment of BHBA infusion rates at achieve and maintain the intended plasma concentration.

Rectal temperature. Rectal temperature was measured during the adaptation period, immediately before the start of infusions, two days after the start of infusion, and hourly during the LPS challenge (8 h).

Milk samples. Foremilk samples (~50 ml) were obtained before morning milking, after teat cleaning from both front quarters (one challenged with LPS and one control) during the adaptation period, immediately before the start of infusion, two days after the start of infusion and hourly during the LPS challenge (8 h). Milk SCC was measured with a DeLaval cell counter (DeLaval International AB, Tumba, Sweden) immediately after sampling.

Udder biopsies. One week before the start of infusions, 48 h after the start of the infusions (before LPS challenge), and at the end of experiment (56 h) udder tissue biopsies were taken from the two rear quarters. Before the biopsies cows were sedated by an intravenous injection of 16 µg/kg of BW of xylazine (Xylazin Streuli ad us. Vet.; G. Streuli & Co. AG, Uznach, Switzerland). Udder tissue (30 to 60 mg) was obtained under local anesthesia with 10 mL Lidocain 2% (Streuli Pharma AG, Uznach), using a biopsy needle with a 12 G and 10 cm

(Bard® Magnum® Core Tissue Biopsy Needle, Türkenfeld, Germany). The samples were placed immediately into RNA stabilization reagent (RNAlater®, Ambion, Applied Biosysthems, Austin, TX), kept at +4 °C for 24 h, and stored at –80 °C until RNA extraction.

Liver biopsies. Liver tissue (60 to 100 mg) was taken one week before the infusions and at 48 h after the start of infusions, under local anesthesia with 10 mL Lidocain 2% (Streuli Pharma AG, Uznach) as described previously (Zarrin et al., 2013), the samples were kept at +4 °C for 24 h in RNA stabilization reagent (RNAlater®, Ambion, Applied Biosysthems, Austin, TX), and stored at –80 °C until RNA extraction.

Laboratory procedures

Udder and liver tissue. Total RNA was extracted from udder and liver tissues with peq GOLD TriFast™ (PEQLAB Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer's protocol. Quantity and purity of RNA was measured by using NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) at 260 nm and 280 nm absorbance waves. Complementary DNA (cDNA) was made by reverse transcription of one µg of total RNA with Moleney Murine Leukemia Virus Reverse Transcriptase RNAase H Minus, Point Mutant (MMLV-RT; Promega Corp., Madison, WI) and random hexamer primers (Invitrogen, Leek, The Netherlands). The mRNA abundances of housekeeping genes (glyceraldehyde 3-phosphate dehydrogenase [GAPDH] and ubiquitin) and target genes related to immune response were measured by real-time quantitative PCR (qPCR) in a Rotor-Gene 6000 rotary analyzer (Corbett Research, Sydney, Australia) and software version 1.7.75, using a master mix consisting of 0.8 µL PCR water, 1.0 µL forward primer (5 pmol), 1.0 µL reverse primer (5 pmol), and 5.2 µL 2x SensiMix plus SYBR-Green. The cycle threshold (CT) values obtained for candidate genes were adjusted according the mean of the housekeeping genes CT according to the following equation: ΔCT = CT (arithmetic mean of housekeeping genes) – CT (target gene). Differences in mRNA abundance during 48 h BHBA infusion in udder and

187 liver were calculated according to the following equation: ΔΔCT = ΔCT [d 2 (48 h)] – ΔCT
188 [d 0 (0 h)]. Differences in mRNA abundance during LPS challenge were calculated for LPS
189 and control quarters separately according to this equation: ΔΔCT = ΔCT [d 3 (8 h)] – ΔCT [d
190 3 (0 h)], as described by Vernay et al. (2012). The primer sequences for housekeeping and
191 candidate genes were used according to previous publications: GAPDH, IL-8, SAA, and
192 tumor necrosis factor-alpha (TNFα) (Wellnitz et al., 2006), ubiquitin, lactoferrin, and
193 inducible nitric oxide synthase (iNOS) (Schmitz et al., 2004), nuclear factor kappa-light-
194 chain-enhancer of activated B cells (NF-κB) (Bruckmaier and Meyer, 2005), Hp (Hiss et al.,
195 2004), and IL-1β (Griesbeck-Zilch et al., 2008). The selected and measured genes, the used
196 primer sequences, and PCR conditions are shown on Table 1.
197
198 *Statistical analysis*
199 Data are presented as means ± SEM and differences were considered significant if $P < 0.05$,
200 and as a tendency if $P < 0.10$.
201 Differences between before and after 48 h BHBA infusion and before and after LPS challenge
202 (delta) were calculated for all recorded parameters. Somatic cell counts were analyzed and are
203 shown at logarithmic scale ($\log_{10}$) to ensure normal distribution of data. Area under the curve
204 was calculated for RT and SCC during the LPS challenge (8 h). Mammary mRNA abundance
205 data were analyzed by using the general linear models (GLM) procedure of SAS (SAS
206 Institute Inc., Cary, NC, USA, 2002-2008, Release 9.2), including treatment (BHBA or NaCl)
207 as fixed effect. Differences between means were evaluated by the Tukey's test. In addition,
208 means of delta values obtained within each treatment were tested for differences from "0".
209 Differences in SCC, and RT between treatments within each time point and between time
210 points within treatments were evaluated by using the ANOVA mixed model procedure of
211 SAS, including treatment (BHBA or NaCl), time, and their interactions as fixed effects and
212 cows as repeated subject.

RESULTS

As reported earlier the mean infusion rate of BHBA during 48 h was 8.1 ± 0.3 µmol/kg/min, and plasma BHBA concentration was maintained at 1.7 ± 0.1 mmol/L in the HyperB group (Zarrin et al., 2013). In the control group plasma BHBA concentration was 0.6 ± 0.1 mmol/L. In response to LPS administration at 48 h of infusion, plasma BHBA started to decrease ($P<0.01$) and the infusion had to be increased ($P<0.001$) to 11.1 ± 1.0 µmol/kg/min to maintain hyperketonemia (Figure 1). Despite increased BHBA infusion rate after LPS administration mean plasma BHBA concentration after LPS challenge (8 h) was not fully maintained and was 1.4 ± 0.1 mmol/L in HyperB. Also in the control group plasma BHBA decreased ($P<0.01$) after LPS administration (0.4 ± 0.1 mmol/L).

Somatic cell counts and rectal temperature

Beta-hydroxybutyrate infusion did not affect RT and milk SCC during 48 h of BHBA infusion (before LPS challenge; data not shown). The subsequent intramammary LPS challenge caused a significant increase in SCC in LPS challenged quarters compared to control quarters in both groups within 3 h after LPS administration (Figure 2). The increase of SCC was lower in the HyperB group than in the control group ($P<0.01$). In response to LPS challenge RT increased similarly in both groups from 38.6 ± 0.0 °C and 38.1 ± 0.2 °C to a maximum of 40.5 ± 0.2 °C and 40.8 ± 0.1 °C to be reached at 6 h and 5 h after LPS administration in HyperB and NaCl, respectively.

Changes of mRNA abundance during BHBA infusion in liver and mammary tissue before LPS challenge

238 Changes of mRNA abundance of candidate genes related to immune response in liver and
239 mammary tissue are shown on table 2. During 48 h of infusion the mRNA abundance of SAA
240 and Hp in the liver increased similarly in both groups.
241 In mammary tissue the mRNA abundance of SAA increased during the 48 h of infusion in
242 HyperB ($P<0.01$) but not in the control group. Haptoglobin mRNA abundance tended to
243 increase in HyperB compare to control group ($P= 0.07$). The mRNA abundance of the other
244 measured candidate genes in the mammary gland did not change during the first 48 h of
245 BHBA and NaCl infusion.
246
247 ***Changes of mRNA abundance in mammary tissue in response to LPS challenge***
248 Changes of mRNA abundance of candidate genes in mammary tissue related to the immune
249 response to LPS are shown on table 3. Intramammary LPS challenge caused an increased
250 mRNA abundance of IL-1β, IL-6, IL-8, IL-10, TNFα, iNOS, NF-κB, Hp, and SAA in the LPS
251 challenged quarters in both treatment groups. The mRNA abundance of IL-6, IL-10, and Hp
252 increased also in the control quarters in both treatment groups. In the HyperB group the
253 increase of mammary mRNA abundance in quarters with LPS administration was more
254 pronounced than in the control group for IL-8 ($P<0.01$) and IL-10 ($P<0.05$), respectively.
255
256
257 DISCUSSION
258 To the best of our knowledge, the present experiment is the first study to investigate the
259 effects of long term hyperketonemia through BHBA infusion on SCC and immune response
260 in dairy cows that were stimulated with intramammary *E. coli* LPS.

261 Unchanged SCC in milk during 48 h of increased BHBA concentrations in blood in the
262 present study was in contrast to a previous study (Duffield et al., 1998) that reported an

association between hyperketonemia and increased SCC after parturition in dairy cows. However, the BHBA infusion represented an additional energy source during already positive energy balance in the present study as compared to the NEB that was reported in the mentioned study. In addition, in the present study the induced hyperketonemia by BHBA infusion was completely different to spontaneous hyperketonemia during NEB that is accompanied by low plasma glucose concentration and high plasma NEFA concentration after parturition (Kessel et al., 2008; van Dorland et al., 2009; Gross et al., 2011).

Consistently with previous studies LPS challenge caused the increase of body temperature (Schmitz et al., 2004; Vernay et al., 2012) and milk SCC (Wellnitz et al., 2011; Vernay et al., 2012). The induced increase of RT confirmed that LPS challenge caused not only a local activation of the immune response but had also a systemic effect (Dinarello, 1991). An increase of SCC is accepted as an indicator of inflammatory responses in the mammary gland (Schukken et al., 2003; Pfaffl et al., 2003; Wellnitz et al., 2011). Interestingly, the increase of milk somatic cells was less pronounced in LPS quarters in HyperB than the control group. This is supported by earlier in vitro studies that illustrated that elevation of BHBA concentration decreased neutrophil chemotactic response in bovine milk leukocytes *in vitro* (Cerone et al., 2007; Klucinski et al., 1988). Therefore, it can be assumed that the diminished increase of SCC in the present study in the HyperB group compared to the control group is related to the negative effects of plasma BHBA concentration on neutrophil recruitment.

Increased hepatic mRNA abundance of SAA and Hp in both treatment groups during 48 h BHBA infusion indicates that the changes are induced by the experimental procedures independent of the applied treatment. It is possible that an increase of SAA and Hp mRNA abundance in the liver is related to experimental stress in both treatment groups. The long term BHBA infusion did not specifically affect hepatic mRNA abundance of these candidate genes. This finding is in agreement with a previous study which illustrated that ketosis does

not affect hepatic Hp and SAA mRNA abundance in dairy cows (Loor et al., 2007), and is in contrast to a study that documented increased serum Hp concentration during ketosis (Stengärde et al., 2008). In contrast to the liver, SAA mRNA abundance in mammary tissue was increased by long term BHBA infusion, and Hp tended to increase in HyperB compared to NaCl during 48 h BHBA infusion in the present study. The different effects of BHBA infusion on SAA and Hp mRNA abundance in the mammary tissue is most likely related to a higher sensitivity of SAA than Hp (Gruys et al., 1993; Alsemgeest et al., 1994; Werling et al., 1996). It can be speculated that the different effect of high plasma BHBA concentration (48 h) on acute phase protein mRNA abundance in the liver and in the udder was related to the role of the liver in the systemic immune response whereas the udder tissue acts mainly locally within the organ.

Increased SAA and Hp mRNA abundance in mammary tissue in response to LPS challenge confirms previous studies that illustrated increased synthesis of SAA (Wellnitz and Kerr, 2004; Bruckmaier and Meyer, 2005; Vernay et al., 2012) and Hp (Hiss et al., 2004) in mammary tissue in response to LPS. Increased udder mRNA abundance of IL-1β, IL-6, iNOS, and TNFα, in quarters that were stimulated by intramammary LPS challenge in both groups is related to immune system activation and was documented in previous studies (Bruckmaier and Meyer, 2005; Wellnitz et al., 2011; Vernay et al., 2012; Hiss et al., 2004). This result demonstrates that increased BHBA concentrations in blood by infusions affect the abundance of measured genes related to immune response in the udder but no in the liver. Increased IL-6, IL-10, and Hp mRNA abundance in LPS quarters and control quarters in both treatment groups illustrated that intramammary LPS stimulation has also an influence on systemic immune response (Schmitz et al., 2004). Modified plasma BHBA concentration induced a more pronounced increase of IL-8 and IL-10 mRNA abundance compared to the control group, in LPS challenged quarters. There is a lack of information about effects of

hyperketonemia on mRNA abundance related to immune response. However, there is evidence that ketone bodies, specifically acetoacetate, increases IL-8, and IL-6 concentration in human U937 and THP-1 monocyte cell lines and human umbilical vein endothelial cells, while the presence of BHBA does not affect these cytokine concentrations (Rains et al., 2011; Jain et al., 2007). IL-8 is a chemokine that is produced by lymphocytes (Gregory et al., 1988), neutrophils (Strieter et al., 1990), and epithelial cells (Skansen-Saphir et al., 1993). IL-8 is involved into the recruitment of neutrophils and activates them (Harda et al., 1994) during mastitis (Barber and Yang, 1998) and mammary epithelial cells secrete IL-8 in response to LPS (Wellnitz and Kerr, 2004). It can be hypothesized that increased IL-8 mRNA abundance after LPS challenge is related to its role in the recruitment of immune cells from blood into milk. As BHBA infusion decreased neutrophil recruitment, the immune system was most likely forced to compensate the deficiency of BHBA effects on immune cell recruitment through the upregulation of IL-8 mRNA abundance. It seems that upregulation of IL-8, and IL-10 in HyperB group compared to the control group reflects negative effects of BHBA on the immune response.

CONCLUSIONS

The results of the present study demonstrate that a long term elevation of plasma BHBA (48 h) affects acute phase proteins in the mammary gland but not in the liver. The immune response to LPS challenge is clearly affected by elevated BHBA plasma concentration what indicates that the frequently observed immunosuppression during spontaneous ketonemia in early lactation and hence increased susceptibility of mastitis during this period is most likely at least in part be directly caused by the elevated concentration of BHBA.

ACKNOWLEDGMENT

This study was supported by a grant of the Swiss National Foundation (grant no. 320030-120317). We would like to thank Claudine Morel, Yolande Zbinden, and Chantal Philipona (Veterinary Physiology, Vetsuisse Faculty, University of Bern, Switzerland) for their support in the laboratory analyses. Authors also would like to thank Dr. Martin Vernay and Dr. Luisa De Matteis for their help during the experiments.

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Figure legends

Figure 1. Beta-hydroxybutyrate (BHBA) infusion rates during 56 h infusion in cows infused with BHBA (HyperB). Plasma BHBA in cows infused with BHBA (HyperB) and cows infused with NaCl as control group (NaCl). Values represent Mean ± SEM. At 48 h of infusion two quarters of both treatment groups were challenged by intramammary LPS.

Figure 2. Somatic cell counts (in log10 scale) in milk after the LPS challenge. Data are means ± SEM in quarters that were challenged with intramammary *E. coli* lipopolysaccharide (LPS) and quarters that received NaCl (C), in cows that were infused with Beta-hydroxybutyrate (HyperB) and cows that were infused with NaCl as control group (NaCl). A, B, C, D and a, b, c, d, e: LPS quarters without common letter are significantly different ($P<0.05$) from base line (time 0 of LPS challenge) in HyperB and control group, respectively. Differences between SCC in LPS quarters ($P<0.05$) in HyperB and control group are indicated with (*).

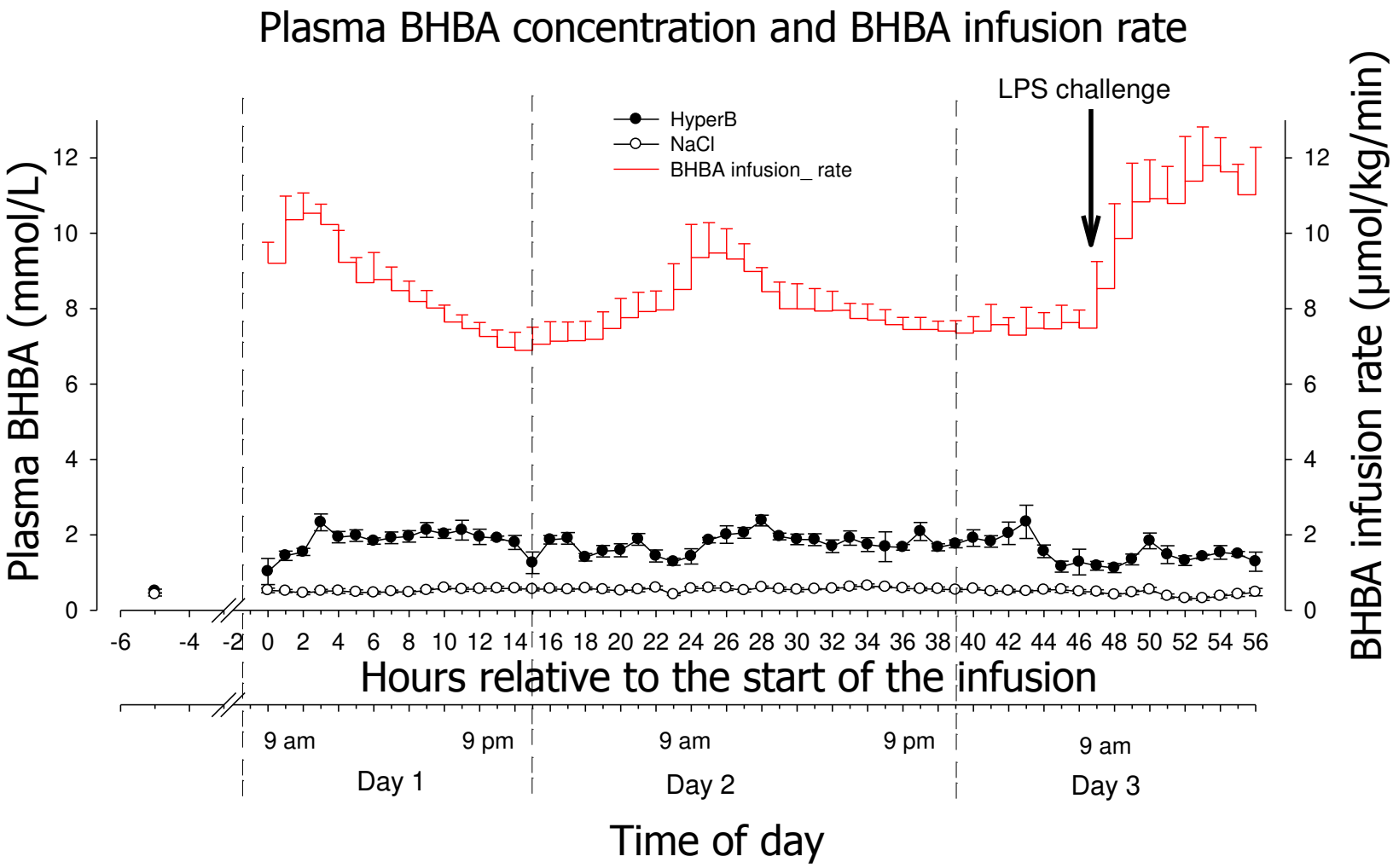
Plasma BHBA concentration and BHBA infusion rate
LPS challenge
HyperB
NaCl
BHBA infusion_ rate
Plasma BHBA (mmol/L)
BHBA infusion rate (µmol/kg/min)
0
2
4
6
8
10
12
-6 -4 -2 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56
Hours relative to the start of the infusion
9 am
9 pm
9 am
9 pm
9 am
Day 1
Day 2
Day 3
Time of day

Figure 1

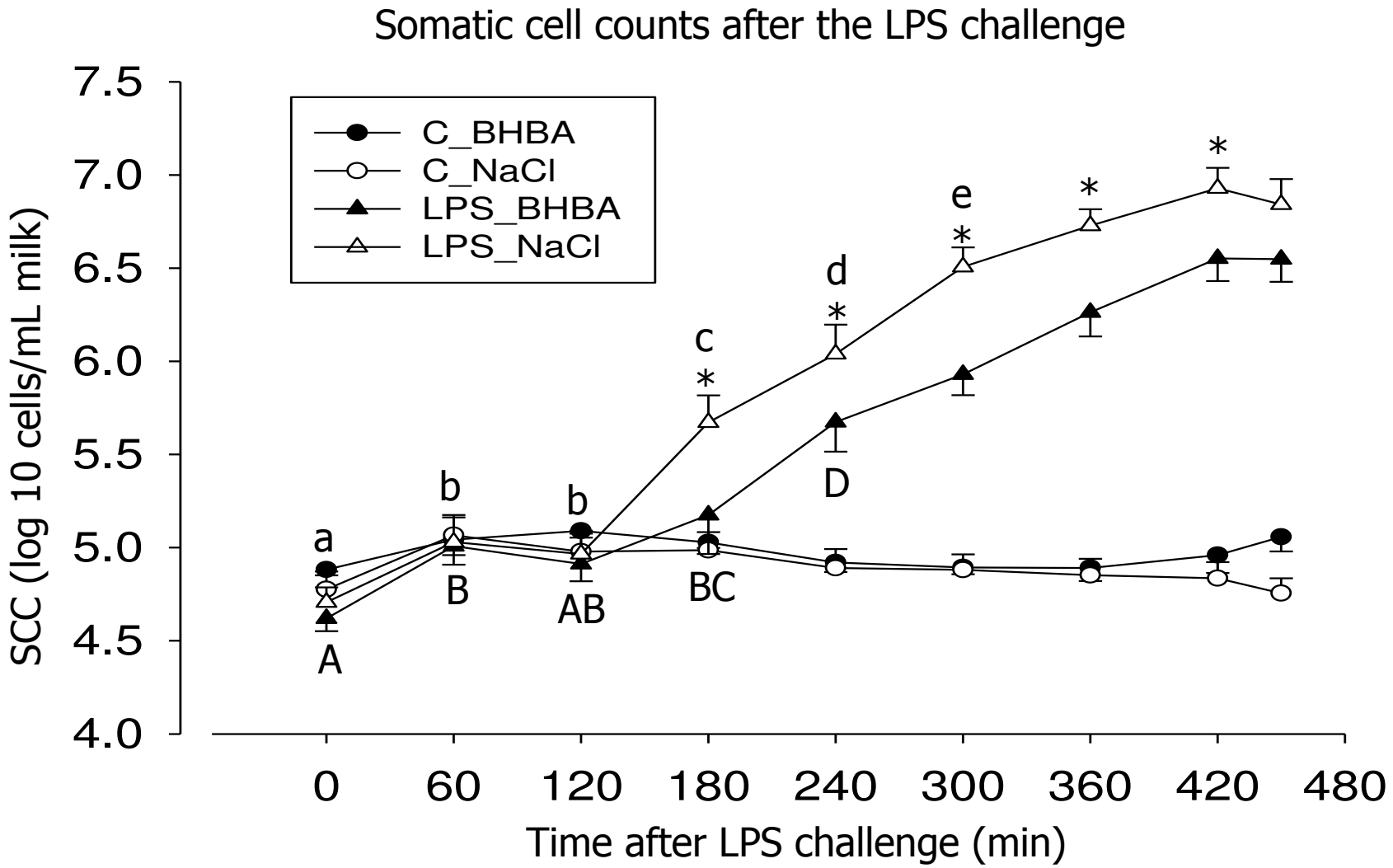
Somatic cell counts after the LPS challenge
C_BHBA
C_NaCl
LPS_BHBA
LPS_NaCl
SCC (log 10 cells/mL milk)
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
0
60
120
180
240
300
360
420
480
Time after LPS challenge (min)
a
b
b
c
d
e
A
B
AB
BC
D
*

Figure 2

Table 1. Polymerase chain reaction primer information (for = forward, rev = reverse), annealing temperature and the PCR product length

Gene[1]		Sequence 5'-3'	Gene Bank accession no.	Annealing temperature, (°C)	Length ,bp
GAPDH	for	GTC TTC ACT ACC ATG GAG AAG G	NM001034034	60	197
	rev	TCA TGG ATG ACC TTG GCC AG			
Ubiquitin	for	AGA TCC AGG ATA AGG AAG GCA T	NM174133	62	198
	rev	GCT CCA CCT CCA GGG TGA T			
IL-1β	for	AGT GCC TAC GCA CAT GTC TTC	M37211	60	114
	rev	TGC GTC ACA CAG AAA CTC GTC			
IL-6	for	CTTCACAAGCGCCTTCACTC	NM_173923.2	62	132
	rev	GTCAGAAGTAGTCTGCCTGG			
IL-8	for	ATG ACT TCC AAG CTG GCT GTT G	AF232704	60	149
	rev	TTG ATA AAT TTG GGG TGG AAA G			
IL-10	for	CCT GGA AGA GGT GAT GCC AC	EU276074	60	133
	rev	GTT TTC GCA GGG CAG AAA GCG			
iNOS	for	ACC TAC CAG CTG ACG GGA GAT	U14640	62	316
	rev	TGG CAG GGT CCC CTC TGA TG			
TNFα	for	CCA CGT TGT AGC CGA CAT C	NM173966	60	155
	rev	CCC TGA AGA GGA CCT GTG AG			
Lactoferrin	for	GGC CTT TGC CTT GGA ATG TAT	L08604	62	338
	rev	ATT TAG CCA CAG CTC CCT GGA G			
NF-κB	for	GCT GGA CCC AAG GAC ATG	NM003998	56	235
	rev	TGG TCT GCT GCA GAG CTG			
Hp	for	GTCTCCCAGCATAACCTCATCTC	AJ271156	55	174
	rev	AACCACCTTCTCCACCTCTACAA			
SAA	for	CCT GGG CTG CTA AAG TGA TC	AF540564	57	184
	rev	TAC TTG TCA GGC AGG CCA G			

[1]GAPDH = glyceraldehyde 3-phosphate dehydrogenase; iNOS = inducible nitric oxide synthase; TNFα = tumor necrosis factor-alpha; NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells; Hp = haptoglobin; SAA = serum amyloid A.

Table 2. Changes of mRNA abundance of genes related to immune response in liver and udder tissue during infusion with beta-hydroxybutyrate (HyperB) or saline (NaCl) during 48h infusion. Delta (differences between before the start of the infusion and 48 h after the start of infusions). Values represent Mean ± SEM.

Parameter[1#]	Group[2]	Delta	ANOVA (P-Value, group)
TNFα	HyperB	0.4 ± 0.1	0.52
	NaCl	0.1 ± 0.4	
Hp	HyperB	3.6 ± 1.3*	0.79
	NaCl	4.0 ± 1.2*	
SAA	HyperB	2.5 ± 0.6*	0.45
	NaCl	1.7 ± 0.7*	
IL-1 β	HyperB	-0.5 ± 0.4	0.32
	NaCl	0.4 ± 0.6	
IL-6	HyperB	0.6 ± 0.6	0.12
	NaCl	-0.4 ± 0.3	
IL-8	HyperB	0.6 ± 0.8	0.47
	NaCl	0.5 ± 0.8	
IL-10	HyperB	-0.3 ± 0.5	0.17
	NaCl	0.5 ± 0.3	
iNOS	HyperB	0.1 ± 0.3	0.45
	NaCl	2.0 ± 1.9	
TNFα	HyperB	0.7 ± 0.5	0.38
	NaCl	0.2 ± 0.3	
Lactoferrin	HyperB	0.6 ± 0.3	0.21
	NaCl	-0.1 ± 0.4	
NF-kB	HyperB	-0.4 ± 0.2	0.64
	NaCl	-0.1 ± 0.4	
Hp	HyperB	0.6 ± 0.4	0.07
	NaCl	-0.3 ± 0.2	
SAA	HyperB	1.4 ± 0.5*	<0.01
	NaCl	-0.7 ± 0.3	

[1]TNFα = tumor necrosis factor-alpha; Hp = haptoglobin; SAA = serum amyloid A; iNOS = inducible nitric oxide synthase; NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells;
[2]HyperB = Hyper beta-hydroxybutyrate group; NaCl = group of cows receiving physiological saline solution.
[#]parameters above the line are related to the liver tissue, and parameters below the line are related to the udder tissue.
*Delta is different from 0 ($P < 0.05$).

Table 3. mRNA abundance of genes related to immune responses in mammary gland of cows infused with beta-hydroxybutyrate (HyperB) or saline (NaCl) during the LPS challenge in LPS and control quarters. Values represent Mean ± SEM.

Parameter[1]	Group[2]	LPS Delta (after LPS – before LPS)	ANOVA (*P*-Value, group)	NaCl Delta (after LPS – before LPS)	ANOVA (*P*-Value, group)
IL-1β	HyperB	5.4 ± 0.9*	0.28	1. 5 ± 1.1	0.59
	NaCl	2.9 ± 1.7*		0.7 ± 0.8	
IL-6	HyperB	5.8 ± 1.2*	0.27	2.2 ± 0.5*	0.75
	NaCl	4.3 ± 0.6*		2.5 ± 0.6*	
IL-8	HyperB	10. 8 ± 0.6*	<0.01	1.5 ± 0.7	0.73
	NaCl	6.5 ± 0.7*		2.1 ± 1.2	
IL-10	HyperB	4. 6 ± 1.1*	0.05	2.2 ± 0.6*	0.26
	NaCl	2.3 ± 0.4*		1.2 ± 0.6*	
iNOS	HyperB	4.6 ± 0.5*	0.47	-0.4 ± 0.4	0.78
	NaCl	4.3 ± 2.6*		-1.7 ± 6.1	
TNFα	HyperB	2.7 ± 1.2*	0.9	0.5 ± 0.9	0.97
	NaCl	2.5 ± 0.7*		0.4 ± 0.6	
Lactoferrin	HyperB	1. 4 ± 1.1	0.86	0.8 ± 0.8	0.81
	NaCl	1.1 ± 0.7		0.6 ± 0.5	
NF-kB	HyperB	1.7 ± 0.4*	0.45	0.1 ± 0.5	0.68
	NaCl	2.3 ± 0.6*		-0.2 ± 0.6	
Hp	HyperB	3.7 ± 0.6*	0.97	1.4 ± 0.6*	0.15
	NaCl	3.8 ± 1.1*		2.5 ± 0.4*	
SAA	HyperB	5.1 ± 1.3*	0.78	1.8 ± 1.6	0.34
	NaCl	5. 6 ± 1.1*		3.4 ± 0.7*	

[1]iNOS = inducible nitric oxide synthase; TNFα = tumor necrosis factor-alpha; NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells; Hp = haptoglobin; SAA = serum amyloid A.
[2]HyperB = Hyper beta hydroxybutyrate group; NaCl = group of cows receiving physiological saline solution.
*Delta is different from 0 ($P < 0.05$).